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ACE inhibition reduces glomerulosclerosis and regenerates glomerular tissue in a model of progressive renal disease

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Today angiotensin II inhibition is primarily used to slow the rate of progression of kidney diseases. There is evidence that these therapies can induce a partial regression of glomerular lesions. However, we do not know yet the extent of sclerotic lesion regression and whether new glomerular tissue is formed to help support the renal function. We used male Munich Wistar Fromter (MWF) rats, an experimental model for progressive kidney disease, to quantify kidney structural lesions upon angiotensin-converting enzyme (ACE) inhibition therapy. Animals were studied at 50 weeks of age, when renal function and structure are severely altered, and after a 10-week observation period, without or with treatment with lisinopril (80 mg/l in drinking water). A group of untreated Wistar rats was used as controls. With age, proteinuria, and serum creatinine worsen, but lisinopril almost normalized proteinuria and stabilized serum creatinine. Serial section analysis of whole glomerular tufts showed that at baseline, glomerulosclerosis affected the entire glomerular population, and that these changes further increased with age. Lisinopril significantly reduced incidence and extent of glomerulosclerosis, with the presence of glomerular tufts not affected by sclerosis (23% of glomeruli). Glomerular volume was not significantly affected by treatment, and glomerular mass spared from sclerosis increased from 46.9 to 65.5% upon treatment, indicating consistent regeneration of glomerular tissue. Lisinopril normalized baseline glomerular transforming growth factor-beta and α-smooth muscle actin overexpression, and prevented worsening of interstitial changes. Hence, ACE inhibition, which is widely used in human kidney disease, may not only halt the progression of renal failure, but also actually induce the regeneration of new renal tissue.

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Five hundred million individuals, world wide,[1] are estimated to suffer some degree of chronic renal disease, and the number may double in the next 10 years. Most of these patients will succumb to cardiovascular events, while others will develop end-stage renal disease (ESRD). This condition will heavily affect medical care in the next years, with an increase in costs, which will be difficult to support in the industrialized countries, while the costs for such programs are simply not applicable in low-income countries. Experimental and human studies focused on the progressive nature of kidney diseases have suggested a common pathogenetic mechanism of kidney function loss, even when the initial insult is removed.[2–5] Currently available therapies help in slowing the rate of progression of renal disease, and effectively retard the need for dialysis.[6] Whether such treatments, based on angiotensin II (AII) antagonism (with angiotensin-converting enzyme (ACE) inhibitors or AII receptor blockade), besides preventing the evolution of renal structural changes,[7,8] are capable of regenerating renal tissue, is difficult to establish in humans.

We have previously demonstrated that amelioration of renal function and regression of structural changes can be induced in susceptible Munich Wistar Fromter (MWF) rats, which genetically develop proteinuria and glomerulosclerosis with age, by the use of drugs that inhibit AII.[9,10] Glomeruli with early sclerotic lesions were remodeled in such a way that sclerotic changes reduced in extensiveness. Others have shown similar findings in models of progressive renal diseases, such as puromycin aminonucleoside nephropathy, aging and 5/6 nephrectomy.[11–14] These studies are based on the observation of single tissue sections, and we have shown that single section evaluation of sclerotic changes underestimates the incidence and extent of sclerosis in experimental and human studies when compared with the complete three-dimensional (3D) reconstruction of the capillary tuft.[15,16] Here, we performed morphometrical analysis of the glomerular capillary tuft, using digital processing of serial section images, for 3D estimation of individual capillary tuft volume and the volume occupied by sclerosis. We investigated whether ACE inhibition therapy

given to MWF rats at the late stage of kidney diseases, when they already manifested heavy proteinuria and severe kidney structural changes, still induces regression of glomerular lesions. Specifically, we quantified to what extent regression of advanced glomerular structural changes, if any, is truly taking place and whether it is related to the regeneration of normal tissue. We also evaluated whether regression of renal structural injury was associated with changes in transforming growth factor-beta (TGF-β) expression and in α-smooth muscle actin (α-SMA) expression, respectively, a profibrogenic cytokine TGF-β,[17] and a marker of myofibroblast differentiation.[18] Age-matched Wistar rats were used as normal controls to take into account aging effects.

RESULTS

As expected, male MWF rats developed massive proteinuria and loss of kidney function during their life, as shown in Figure 1. At the age of 50 weeks, urinary protein excretion averaged 571 ± 62 mg/24 h (MWF 50W) and further increased with age in the MWF 60W group (807 ± 166 mg/24 h). In animals given the ACE inhibitor (MWF + LIS), urinary protein excretion almost normalized in the 10-week treatment period, averaging 120 ± 56 mg/24 h by the end of the treatment (i.e. 60 weeks of age). Untreated MWF rats also showed elevated serum creatinine (1.22 ± 0.15 and 1.92 ± 0.77 mg/dl, respectively, at 50 and 60 weeks of age, $P < 0.01$). This rise in serum creatinine was completely prevented by the ACE inhibitor treatment. In this group of rats, serum creatinine remained stable with time, being comparable at 50 and 60 weeks of age.

In line with previous reports,[19] at conventional light microscopy, glomerular capillary tufts in MWF were frequently affected by segmental or global sclerotic changes already at 50 weeks of age, as reported in Table 1 and in Figure 2. Glomerulosclerosis (GS) index averaged 3.4 (range 2.7–3.7) and 3.7 (range 3.6–4.0), respectively, in untreated MWF rats at 50 and 60 weeks of age, values close to the upper limit of this parameter. In animals treated with lisinopril, GS index was significantly lower (2.9, range 1.7–3.4, $P < 0.05$) than that observed in untreated animals at the same age (60 weeks) and numerically lower than that estimated at 50 weeks of age (see representative Figure 2). The normal Wistar rat at 60 weeks of age was also affected by sclerosis, but to a much

Table 1 | Structural changes of kidney tissue in MWF and in Wistar rats

Age	MWF 50W 50 weeks	MWF 60W 60 weeks	MWF+LIS 60 weeks	Wistar 60 weeks
GS Index (score)	3.4 (2.7–3.7)	3.7 (3.6–4.0)	2.9* (1.7–3.4)	1.1°° (0.8–1.6)
Interstitial Volume (%)	32.2 ± 5.3	39.6 ± 3.3#	31.0 ± 4.5++	9.9 ± 0.3°°
Atrophic tubuli (score)	2.5 (2–3)	2.5 (1–3)	2.0 (1–2)	0.8°° (0–1)
Interstial inflammation (score)	3.0 (3–3)	3.0 (2–3)	3.0 (3–3)	1.0°° (0–1)
Interstial matrix (score)	3.0 (2–3)	3.0 (2–3)	2.5 (2–3)	1.3°° (1–2)

GS=glomerulosclerosis; LIS=lisinopril treated from 50 to 60 weeks of age.
Data are expressed as median (range) or mean ± s.d.
*$P < 0.05$ vs MWF rats at 50 and 60 weeks of age, °°$P < 0.01$ vs all MWF groups, #$P < 0.05$ vs MWF rats at 50 weeks of age, ++$P < 0.01$ vs MWF rats at 60 weeks of age.

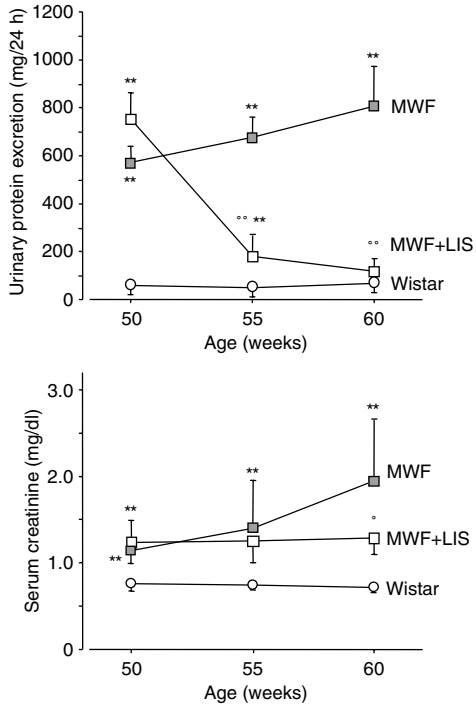

Figure 1 | Urinary protein excretion and serum creatinine in untreated and in lisinopril-treated MWF rats, and in control Wistar rats, from 50 to 60 weeks of age. **$P < 0.01$ vs Wistar rats at same age, °$P < 0.05$ vs MWF rats at same age and °°$P < 0.01$ vs MWF rats at same age.

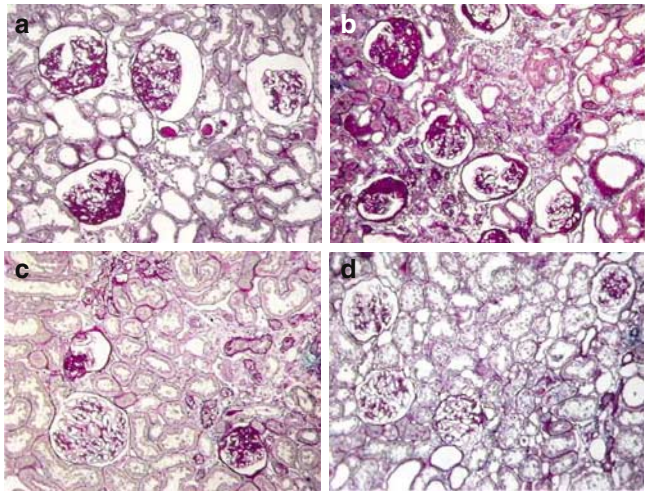

Figure 2 | Representative microphotographs of kidney tissue in untreated MWF rats at (a) 50 and (b) 60 weeks of age, (c) in MWF rats treated with lisinopril from 50 to 60 weeks of age, and (d) in control Wistar rats at 60 weeks of age. Periodic acid-Schiff staining at original magnification × 100.

lesser degree (mean GS index 1.1, range 0.8–1.6). Although these results suggest a tendency of reversal of GS upon treatment, they do not provide the actual change in sclerosis volume and, eventually, the extent of capillary tissue regenerated, if any, by the treatment. Thus, we performed serial section analysis in 100 randomly selected glomeruli per each MWF group ($n = 10$–14 glomerular tuft in each animal), with complete reconstruction of the entire capillary tuft. As shown in Figure 3, mean volume of the glomerular tuft did not change significantly in untreated animals between 50 and 60 weeks and was not significantly altered by lisinopril treatment. On the contrary, the percentage of glomerular capillary tuft occupied by sclerosis averaged $0.43 \pm 0.10\,\mu m^3 \times 10^{-6}$ in untreated MWF 50W group, and significantly increased to $0.77 \pm 0.04\,\mu m^3 \times 10^{-6}$ ($P < 0.01$) with age (MWF 60W), while in animals treated with lisinopril (MWF + LIS), sclerosis volume averaged only $0.29 \pm 0.1\,\mu m^3 \times 10^{-6}$, a value significantly lower than even that calculated in MWF at the starting of the treatment (MWF 50W, $P < 0.01$). Our 3D morphological analysis of individual glomeruli allowed to estimate the incidence and extent of sclerotic change in the populations of reconstructed glomeruli. The percentage of each capillary tuft volume affected by sclerosis is plotted in Figure 3. At 50 weeks (MWF 50W), all but one glomerulus were affected by some degree of sclerosis and a few were completely sclerosed. At 60 weeks of age, in untreated rats (MWF 60W), the sclerosis was more extensive (see Figure 3), and a higher number of glomeruli were completely sclerosed (about 30%). Lisinopril treatment reversed the pattern of GS. Thus, the percentage of tuft volume affected by sclerosis was lower in treated rats than in MWF rats at 50 weeks. In detail, 23% of the glomeruli of treated animals at 60 weeks of age were not affected by sclerosis at all, and the percentage of capillary tuft affected by sclerosis was reduced by 34.9% (from 53.1 to 34.5% in MWF 50W and MWF + LIS, respectively). On the other side, considering the volume of the capillary tuft not affected by sclerosis, this averaged $0.53 \pm 0.03\,\mu m^3 \times 10^{-6}$ in MWF rats at 50 weeks and increased to $0.74 \pm 0.05\,\mu m^3 \times 10^{-6}$ in treated animals 10 weeks later, with an average increase of 40%. Of note, on the contrary, untreated rats lost almost 70% of normal glomerular volume in the 10 week observation period. The pattern of these changes with age (incidence and extent of sclerosis), and the effect of treatment is visualized in Figure 4. In the figure, two surfaces are represented for each glomerular tuft. The gray color surfaces represent the outer part of a glomerular capillary tuft, while the red surfaces represent areas of GS. As shown in the pictures, GS increased in extent in untreated animals from 50 to 60 weeks of age, while in treated animals (MWF + LIS), there appears to be a regression of sclerotic lesions (Figure 4).

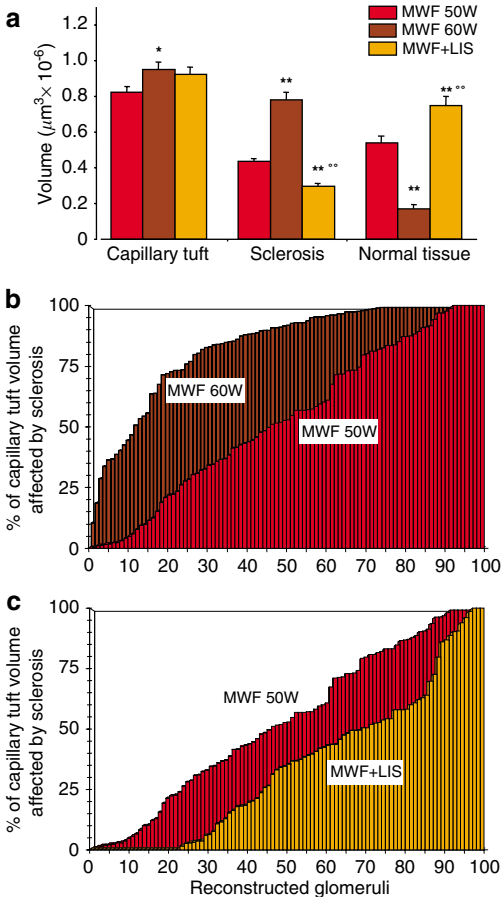

Figure 3 | (a) Average volume of individual glomeruli, sclerosed and nonsclerosed glomerular mass, as reconstructed from untreated (MWF 50W and MWF 60W) and lisinopril-treated rats. Effect of aging on the percentage of glomerular capillary tuft affected by sclerosis in 100 glomeruli reconstructed from MWF rats at 50 and 60 weeks of age (**b**; values for each glomerular capillary were calculated from serial section analysis and ordered increasingly for each group). (**c**) Effect of lisinopril treatment on the percentage of tuft volume affected by sclerosis. *$P < 0.05$ vs MWF 50W, **$P < 0.01$ vs MWF 50W, °°$P < 0.01$ vs MWF 60W.

Besides glomerular, tubular structure and interstitial volume were also markedly altered in MWF rats. Interstitial volume increased significantly in MWF rats over normal controls already at 50 weeks and further rose at 60 weeks, as shown in Table 1. Lisinopril prevented interstitial volume expansion with age. In treated animals, interstitial volume was significantly lower ($P < 0.01$) than that in untreated MWF rats at the same age, and comparable to untreated MWF rats at 50 weeks. Also, tubular atrophy and interstitial inflammation were present in MWF as compared to Wistar animals at 50 weeks (see Table 1). Despite the lower volume density of interstitial area, score for tubular atrophy and interstitial inflammation failed to detect statistically significant differences between treated and untreated animals at 60 weeks.

Morphological alterations of kidney structure in MWF rats were associated with the abnormal expression of TGF-β (as reported in Figure 5 and in Table 2) in glomerular tufts already at 50 weeks, which further increased with age. Glomerular TGF-β expression was normalized in MWF rats by lisinopril treatment, being comparable to that of normal

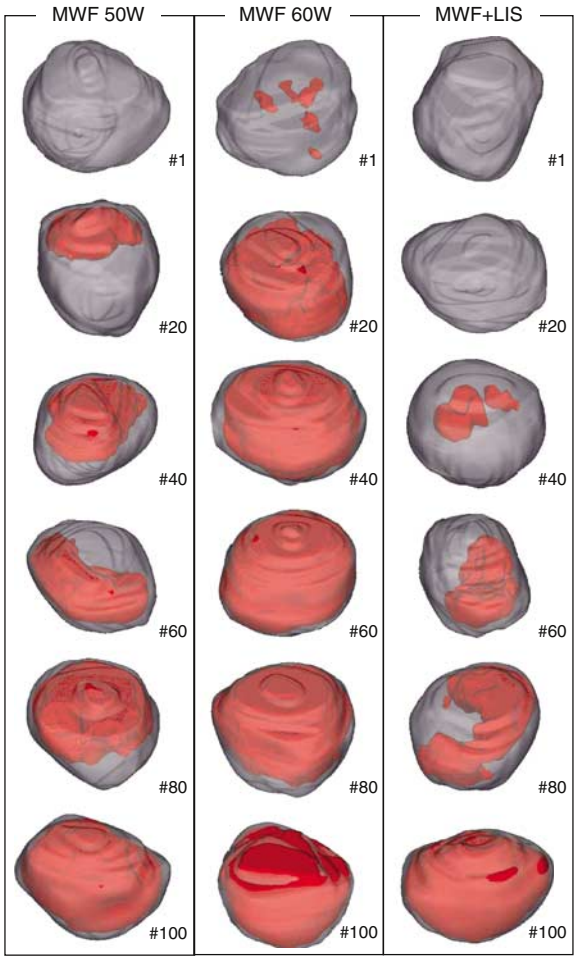

Figure 4 | Visual representation of the distribution of sclerotic areas within the capillary tuft in MWF 50W, MWF 60W and MWF + LIS group. After digital reconstructions of 100 glomerular tufts with the corresponding sclerotic areas, the population was ordered by the percentage of sclerotic volume and six glomeruli were represented. #Position of the glomeruli in the ordered sequence. Glomeruli from treated animals show actual decrease in volume affected by sclerosis, as compared to baseline observation. On the contrary, in untreated animals at 60W, the damage extended to almost entire glomerular mass.

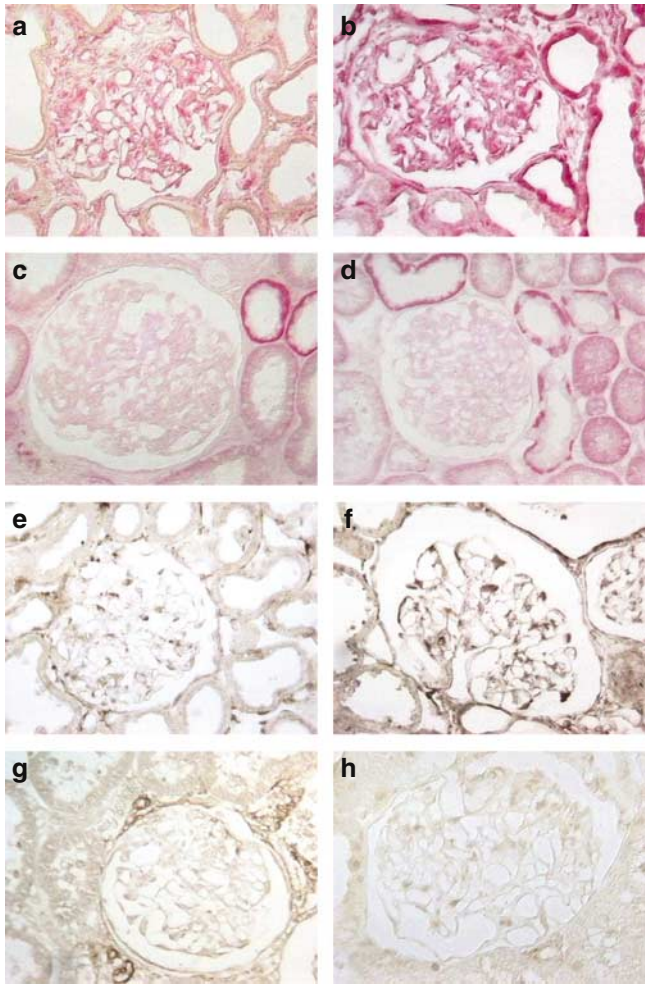

Figure 5 | Representative images of immunohistochemistry performed (a–d) with anti-TGF-β antibody and (e–h) with anti-α-SMA antibody in untreated MWF rats (a and e) at 50 and (b and f) at 60 weeks of age, in MWF rats treated with lisinopril from (c and g) 50 to 60 weeks of age, and (d and h) in control Wistar rats. Original magnification $\times$ 250.

Wistar rats. TGF-β expression in interstitial area was comparable between MWF rats and Wistar rats at 50 weeks. With age, this TGF-β expression numerically increased, but the difference did not reach statistical significance. In treated animals, TGF-β expression was comparable to that of the control group. α-SMA expression (see Figure 5 and Table 2) localized in glomerular capillaries and interstitial area at 50 and 60 weeks of age in MWF rats, while positive staining was only occasionally found in the glomeruli of control Wistar rats. Lisinopril normalized glomerular α-SMA expression in MWF rats, whose levels were comparable to control Wistar rats, without affecting α-SMA expression in interstitial area. TGF-β and α-SMA staining were completely abrogated, omitting the primary antibody (data not shown).

Table 2 | Expression of TGFβ and α-SMA in glomeruli and cortical interstitium in MWF and in Wistar rats at 60 and 50 weeks of age

Age	MWF 50W 50 weeks	MWF 60W 60 weeks	MWF+LIS 60 weeks	Wistar 60 weeks
TGF-β expression				
Glomeruli	1.0	3.0	$0.3^{++, \#\#}$	0^{**}
(score)	(1–2)	(2–3)	(0–1)	(0–0)
Interstitial area	2.0	3.0	2.0^{++}	2.0^{++}
(score)	(1–2)	(3–3)	(2–2)	(2–2)
α-SMA expression				
Glomeruli	1.5	2.0	0.5	$0.5^{\#, ++}$
(score)	(1–2)	(1–3)	(0–1)	(0-1)
Interstitial area	1.5	2.0	$1.0^{\#, +}$	0°
(score)	(1–2)	(1–2)	(1–1)	(0-1)

Data are expressed as median (range).
$^{**}P<0.01$ vs MWF rats at 50 and 60 weeks of age, $^{\circ}P<0.05$ vs all MWF groups, $^{*}P<0.05$ vs MWF rats at 50 weeks of age, $^{\#\#}P<0.01$ vs MWF rats at 50 weeks of age, $^{+}P<0.05$ vs MWF rats at 60 weeks of age and $^{++}P<0.01$ vs MWF rats at 60 weeks of age.

DISCUSSION

The present results show that, in a model of spontaneous renal disease leading to proteinuria, GS, and loss of kidney function,[20] ACE inhibition significantly reduced glomerular sclerotic lesions and promoted the formation of new normal capillary tissue. That glomerular structural changes can be ameliorated by drugs that antagonize the biological action of AII has been shown consistently in different experimental models.[10–14] However, previous studies did not allow to quantify the effective reduction in the extent of sclerotic volume and, more importantly, they could not quantify whether some degree of glomerular capillary regeneration is induced by the therapy. Studies aimed at establishing whether these therapies can effectively promote the formation of intact tissue are of paramount importance to predict if regression of structural changes will be associated with amelioration of renal function in the long term.

Using 3D analysis of individual capillary tufts, here we directly quantified total capillary tuft volume and the volume affected by sclerosis. Total capillary tuft did not change significantly with ACE inhibition, and this is in line with previous observation in the renal ablation model in which ACE inhibition only numerically reduced capillary tuft volume.[14] At variance, our results show that the volume of capillary tuft affected by sclerosis was significantly reduced by treatment. The difference between these two mean volumes indicates that sclerosis was effectively reabsorbed and, on the other hand, that a consistent amount of glomerular tissue regained normal structure, suggesting neoformation of glomerular capillary segments. In detail, at baseline, sclerotic changes affected the entire glomerular population, all but one glomerulus showed different degrees of sclerosis (see Figure 3). Upon lisinopril treatment, the volume extent of these changes uniformly decreased with more relevant effects in glomeruli affected by less than 25% in tuft volume. Actually, an important percentage (23%) of glomeruli were no longer affected by sclerosis after the treatment. This observation strongly supports the hypothesis that small sclerosis changes can be completely regressed by inhibition of ACE. In glomeruli more heavily affected by sclerosis, a significant reduction of the sclerosis volume was also observed, while, as expected, glomeruli occupied by sclerosis for more than 80% of their tuft volume did not benefit from treatment. Considering the entire sample of the glomerular population used for morphometrical analysis, the total sclerotic volume was reduced by 34.9%. This result is visualized by the images reported in Figure 4. On the other hand, taking into consideration that mean glomerular volume did not change significantly during treatment, our calculations showed that the volume of normal capillary tissue increased by 40% during this period.

The mechanisms by which AII antagonism induced regression of sclerotic scar tissue and increased the volume of nonsclerosed capillary tuft are behind the aims of the present study. However, previous data reported in literature indicate some potential mechanisms. Among them, a decreased mRNA and protein expression of plasminogen activator inhibitor-1 (PAI-1) by ACE inhibition in an aging model of nephropathy in the rat and in the renal ablation model has been reported to characterize remodeling of GS.[12] This observation indicates direct involvement of matrix protein degradation in the process of sclerosis regression. Here, we focused on the renal expression of TGF-β and α-SMA. We observed an age-related increase in glomerular expression of both proteins in MWF rats, which may explain the massive fibrosis and cell proliferation we have previously documented in this model by morphometrical analysis.[19] The fact that ACE inhibition favorably affected TGF-β and α-SMA expression at glomerular level may suggest that regression of sclerotic lesions is also related to reduction in extracellular matrix production and accumulation by glomerular cells as well as to a favorable effect on glomerular cell differentiation.

Regarding the possible mechanisms that may induce repair of glomerular capillaries, our present data cannot help in identifying them. Recent data in the renal ablation model[14] would suggest that ACE inhibition in this model is not associated with proliferation of resident glomerular cells. However, Asano and co-workers,[21] recently demonstrated that when podocytes are severely damaged, proliferating parietal epithelial cells migrate onto the visceral site, mimicking proliferating podocytes. Specific studies on glomerular cells and related changes in glomerular capillary architecture are needed to gain more information on this issue. However, among possible candidates that may play a role in this form of tissue repair, there are progenitor cells of the bone marrow, which have been shown to interact with glomerular cells.[22,23]

In conclusion, this study demonstrates that late-stage intervention with ACE inhibitors affords consistent regression of glomerular injury and regeneration of nonsclerosed tissue in a genetic model of renal disease. Besides indicating the possibility to induce the regression of glomerular damage, our results may have important implications for the treatment of progressive chronic glomerulopathies. If these results can be successfully transferred to the clinical condition, they may allow to effectively reduce the use and the costs of life-long renal replacement therapies. On the basis of these evidences, it is of crucial importance to investigate cellular and molecular mechanisms that underlay this process, with the aim to extend, in incidence and duration, the beneficial effects of these treatments.

MATERIALS AND METHODS

Study design

Twenty-five male MWF rats and four Wistar rats (Charles River Italia S.p.A., Calco, Italy) were used. MWF rats belonging to our colony[20] were established to have a high number of surface glomeruli, previously used for micropuncture studies.[24] Animals were divided into four groups. The first group, consisting of MWF rats ($n = 9$), was followed until the age of 50 weeks (MWF 50W) without treatment and was used at this age to evaluate serum creatinine, protein excretion and to perform morphological and

morphometrical analysis of kidney tissue. The second group of MWF rats ($n = 7$) was followed until the age of 60 weeks (MWF 60W), and was similarly left untreated until this age, then used for renal functional and structural evaluation. MWF rats of the third group ($n = 9$) were left untreated until the age of 50 weeks and then treated with a high dose of the ACE inhibitor lisinopril in drinking water (80 mg/l) for 10 weeks (MWF + LIS). After treatment (i.e. at the age of 60 weeks), animals were used for kidney function and structural evaluation. A group of Wistar rats left untreated up to 60 weeks of age was used as the control group. Serum creatinine and protein excretion were periodically measured during the observation period using conventional methods. All animals were maintained in a temperature-controlled room, regulated with a 12-h light/dark cycle, and allowed free access to water and food (standard rat chow with 20% in protein). Animal care and treatment were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (EEC CD 86/609, OJL 358, 1987; DL n 116, GU, 18/2/1992, Circ. N. 8, GU, 14/7/1994; Guide for the Care and Use of Laboratory Animals, US NRC, 1996).

Morphological evaluations

Kidney tissue was obtained and processed for morphological evaluation as described in detail previously.[10] Briefly, after anesthesia, animals were opened through a mid-line incision, and the abdominal aorta was cannulated below the renal arteries, with a catheter connected to a pressure transducer (Battaglia Rangoni, Bologna, Italy). The left kidney was perfused with saline solution at the measured arterial pressure for 5 min, and then with a solution of 1.25% glutaraldehyde in 0.1 mol/l cacodylate buffer. The kidney was then removed, weighted and postfixed in Dubosq–Brazil fluid. After paraffin embedding, sections 3 μm in thickness (Ultrotome V, LKB, Bromma, Sweden) were stained with Masson's trichrome, hematoxylin and eosin, and by the periodic acid-Schiff techniques, as described previously.[10]

The incidence and extent of glomerular and tubular structural lesions were estimated at light microscopy, with conventional single section evaluation and at single glomerular capillary level. For conventional evaluation, at least 100 glomeruli were examined for each animal, including the superficial and juxtamedullary cortical area. Each glomerulus was scored according to the extent of sclerotic changes consisting of matrix deposition, capillary occlusion and capillary tuft adhesion to Bowman's capsule.[10] Score was assigned to 0 in the absence of sclerosis, to 1 for changes affecting less than 25% of the glomerular area, to 2 and 3 for lesions affecting 25–50 and 50–75% of the tuft, respectively, and to 4 for lesion exceeding 75% of the tuft. The average GS index in each animal was then calculated as weighted mean. For single glomerular volume estimation, serial section analysis with digital image processing and 3D reconstruction was used, with some modifications of previously described methods.[15] Briefly, for each animal, a series of serial sections of kidney cortical tissue (3 μm in thickness) was obtained and the middle section of the series was labeled as 'starting section'. In the starting section, 15–20 glomeruli were randomly identified (irrespective of their size and the presence or extent of sclerosis), and used for acquisition of digital images at light microscopy of the entire glomerular capillary tuft, above and below the starting section, until the glomerular tuft disappeared in the following section. Digital images were processed with interactive tools (Image J, http://rsb.info.nih.gov/ij) to outline separately the outer polygon of the capillary tuft and, if present, the sclerotic region of the glomerular tuft. The coordinates of each pixel of these outlines were stored in the computer memory, and used to calculate and graphically represent the capillary tuft area and the sclerosis area in each section. For calculation of capillary tuft and sclerosis volume, the sum of the respective surface areas along the entire series of images acquired for each individual glomerulus was performed, and multiplied by section thickness. In total, 100 glomeruli were analyzed for each MWF rat group (MWF 50W, MWF 60W and MWF + LIS). For visualization of the fraction of the capillary tuft volume occupied by sclerosis at single glomerular level, we developed a computer program in C++ using the VTK libraries (Visualization Tool Kit, http://public.kitware.com/). This program allowed us to automatically generate 3D views of a hypothetical surface, wrapping the glomerular capillary tufts as a transparent gray surface, and the volume occupied by sclerotic changes as solid red surfaces.

We also determined volume density of the cortical interstium by digital morphometrical analysis. Briefly, in a single kidney section for each animal, 30 systematically selected fields in the renal cortex were digitized, and a 10×10 line orthogonal grid was digitally overlayed (using the digital image-processing program Image J, http://rsb.info.nih.gov/ij/). Volume density was calculated as the ratio between grid points hitting interstitial space and total number of grid points contained in the renal cortex. Image acquisition and point counting were repeated until the expected probable error of the mean volume density was less than 5%.[25] Tubular structural changes were evaluated by semiquantitative scores. Tubular atrophy, interstitial matrix accumulation and inflammation were graded from 0 to 3+ (0 = no changes; 1+ = changes affecting less than 25% of the sample; 2+ = changes affecting 25 to 50% of the sample; 3+ = changes affecting more than 50% of the sample). At least 10 fields per kidney slice were examined for histological scores at low-power magnification. All tissue sections were analyzed by the same pathologist in a single-blinded manner.

Immunohistochemical analysis

TGF-β accumulation was detected on paraffin-embedded sections using an alkaline phosphatase-Fast Red technique.[26] The slides were heated twice for 5 min in 0.01 mol/l sodium citrate buffer, pH 6.0, in a microwave oven at an operating frequency of 2450 MHz and 600-W power output. They were allowed to cool for 15 min and then rinsed in distilled water twice and in phosphate-buffered saline (PBS) for 5 min. Sections were blocked with PBS and 1% bovine serum albumin (BSA), then incubated overnight at 4°C with the primary antibody, a polyclonal rabbit anti-TGF-β rat (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing in PBS, biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA, USA) was applied for 30 min at room temperature. Sections were then washed with PBS and incubated with alkaline phosphatase-conjugated streptavidin (1:50; Roche Molecular Biochemicals, Mannheim, Germany) for 30 min at room temperature, followed by washes with PBS and by development with Fast Red substrate (Roche Molecular Biochemicals, Mannheim, Germany). Sections were finally mounted using an aqueous mounting medium (Bio-Optica, Milan, Italy).

The myofibroblast marker, α-SMA, was evaluated by a mouse monoclonal antibody (1:400; Sigma Co., St Louis, MO, USA). To serve this purpose, Dubosq–Brazil fixed and paraffin-embedded kidney sections were deparaffinized, rehydrated and incubated for 30 min with 0.3% H_2O_2 in methanol to quench endogenous peroxidase. Then the tissue was permeabilized in 0.1% Triton X-100 in PBS 0.01 mol/l, pH 7.2, for 30 min and the sections were

blocked with 1% PBS/BSA. Primary antibody was added overnight at 4°C, followed by the secondary antibody consisting of biotinylated sheep anti-mouse IgG diluted 1:100, (Chemicon Temecula, CA), avidin–biotin peroxidase complex (ABC) solution, and finally developed with diaminobenzidine. Negative controls were obtained by omitting the primary antibody on a second section present on all glass slides. The staining for TGF-β and α-SMA was assessed by a semiquantitative evaluation at light microscopy. A score was assigned to each glomerulus observed accordingly to the following pattern: 0, absence of staining of the glomerular tuft; 1, weak staining; 2, staining of moderate intensity; and 3, strong staining of the glomerular tuft. All renal tissue sections were analyzed by the same pathologist, in a single-blinded manner.

Statistical analysis

Statistical analysis was performed using the computer software Stat View® (Abacus Concept Inc., Berkeley, CA). Data were analyzed using the analysis of variance with Bonferroni/Dunn test to assess statistical significance of specific comparisons. Nonparametric data, such as morphological scores, were analyzed with the Kruskal–Wallis test, as specified. The level of $P<0.05$ was considered statistically significant.

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